B.30 CHRONIC TOXICITY TEST

1. METHOD

1.1 Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their life span. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

Test conditions

Experimental animals

The preferred species is the rat. Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20% of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

Number and sex

For rodents at least 40 animals (20 female and 20 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

For non-rodents a smaller number of animals, but at least four per sex per group, is acceptable.

Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit definite signs of toxicity without causing excessive lethality. The lowest dose level should not produce and evidence of toxicity .

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking watet or mixed in the diet it should be continuously available.

Controls

A concurrent control group which is identical in every respect to the treated groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should also be used. The negative control group is treated in the same manner as the test groups except that the animals are not exposed to the test substance or any vehicle.

Route of administration

The two main routes of administration are oral and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

The use of the dermal toute presents considerable practical problems. Chronic systemic toxicity resulting from percutaneous absorption can normal1y be inferred form the results of another oral test and a knowledge of the extent of percutaneous absorption derived from preceding percutaneous toxicity tests.

Oral studies

Where the test substance is absorbed from the gastrointestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred unless there are contra-indications.

The animals may receive the test substance in the diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should also be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chamber. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure, and by the need for more complicated (and reliable) aerosol and vapour , generation and monitoring techniques.

Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in al1 respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to

ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the chamber .

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;
(ii) Concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15% of the mean value;
(iii) Temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2°C, and the humidity within the chamber at 30 to 70% , except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;
(iv) Particle size measurements: particle-size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of study

The duration of the period of administration should be at least 12 months.

Procedure

Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, for example necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be made to detect onset and progression of all toxic effects as well as to minimize loss due to disease, autolysis or cannibalism.

Clinical signs, including neurological and ocular changes as well as mortality, should be recorded for all animals. Time of onset and progression of toxic conditions, including suspected tumours, should be recorded.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter. Food intake should be determined weekly during the first 13 weeks of the study, and then at approximately three-monthly intervals unless health statuS or body weight changes dictate otherwise.

Haematological examination

Haematological examination (e.g. haemoglobin content; packet cell volume, total red blood cells, total white - blood cells, platelets or other measures of clotting potential) should be performed at three months, six months, and thereafter at approximately six-month intervals and at termination on blood samples collected form all non-rodents and from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents.

If clinical observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples from the animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from aIl non-rodents and from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made for either individual animals or on a pooled sample/sex/group for rodents:

-appearance: volume and density for individual animals,
-protein, glucose, ketones, occult blood (semi.quantitatively),
-microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

-total protein concentration,

-albumin concentration,

-liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([1]) activity and glutamic oxaloacetic transaminase ([2]) activity), gamma glutamyl transpeptldase, ornithine decarboxylase,

-carbohydrate metabolism such as fasting blood glucose,

-kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals, for differential blood counts. All grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations wirh the microscopic findings.

All organs and tissues should be preserved for histopathological examination. T his usually concerns the following organs and tissues: brain ([3]) (medullaipons, cerebellar correx, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([3]), spleen, kidneys ([3]), adrenals ([3]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads ([3]), accessory genital organs, female mammary gland, skin, musculature, peripheral, nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes. Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, because it may give significant guidance to the pathologist.

Histopathology

All visible changes, particularly tumours and other lesions occurring in any organ should be examined microscopically. In addition, the following procedures are recommended:

(a) Microscopic examination of all preserved organs and tissues with complete description of all lesions found in:

1. all animals that died or were killed during the study;
2. 2. all of the high-dose group and controls;

(b) Organs or tissues showing abnormalities caused, or possibly caused, by the test substance are also examined in the lower-dose groups;

([1]) Now known as serum alanine aminotransferase.
([2]) Now known as serum aspartate aminotransferase.
([3]) These organs, from ten animals per sex per group for rodents and all non-rodents, plus thyroid (with parathyroids) for all non-rodents, should be weighed.

(c) Where the result of the test gives evidence of substantial reduction of the animals' normal lifespan or the induction of effects that might affect a toxic response, the next-lower dose level should be examined as described above;

(d) Information on the incidence of lesions normally occurring in the strain of animals used, under the same laboratory conditions, i.e. historical control data, is indispensible for correctly assessing the significance of changes observed in treated animals.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

-species, strain, source, environmental conditions, diet,
-test conditions:

Description of exposure apparatus:
including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:
these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;
(b) temperature and humidity of air;
(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);
(d) nature of vehicle, if used;
(e) actual concentrations in test breathing zone;
(f) median particle sizes (where appropriate),

-dose levels (including vehicle, if used) and concentrations,
-toxic response data by sex and dose,
-no-effect level,
-time of death during the study or whether animals survived to termination,
-description of toxic and other effects,
-the time of observation of each abnormal sign and its subsequent course,
-food and bodyweight data,
-ophthalmological findings,
-haematological tests employed and all results,
-clinical biochemistry tests employed and all results (including results of any urinalysis),
-necropsy findings,
-a detailed description of all histopathological findings,
-statistical rreatment of results where possible,
-discussion of the results,
-interpretation of the results.

3.2. Evaluation and interpretation

See General Inrroduction Part B.

4. REFERENCES

See General Introduction Part B.